

Applicants: Cravatt et al.  
Serial No.: 09/738,954  
Filed: December 15, 2000

PATENT  
Attorney Docket No.: SCRIP1210-2

*rule 126*  
*51*  
*46* (New) A method according to Claim 25 wherein said group of related enzymes is metalloenzymes.

*52*  
*47* (New) A method according to Claim 26 wherein said group of related enzymes is redox enzymes.—  
*617*  
*claims*

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REMARKS

Support for the amendments for clarity and new claims is found throughout the specification and claims as originally filed. The amendments to Claims 11-13, 15, 17-19 find support in, for example, original Claims 11-13, 15, 17-19, respectively, as well as pages 23- 41 of the specification and Examples 4, 5, 6 and 7. The amendments to Claims 21-26 find support in, for example, original Claims 21-26, respectively. New Claim 27 finds support in, for example, original Claims 12, 17, and 18. New Claim 28 finds support in, for example, original Claims 13, 17 and 18. New Claim 29 finds support in, for example, original Claims 14, 17 and 18. New Claim 30 finds support in, for example, original Claims 15, 17 and 18. New Claim 31 finds support in, for example, original Claims 16, 17 and 18. New Claim 32 finds support in, for example, original Claim 17. New Claims 33-36 find support in, for example, pages 27-40 of the specification. New Claims 37-41 find support in, for example, Examples 1-7. New claims 42-47 find support in, for example, original claims 21-26, respectively. No new matter is added. Applicants respectfully request entry of these amendments and reconsideration of the application.

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If the Examiner would like to discuss any of the issues raised in the Office Action,  
Applicant's representative can be reached at (858) 677-1456. While it is not believed that any  
fees are due, if the PTO finds any additional fees due, or make any credits, to Deposit Account  
No. 50-1355.

Respectfully submitted,

Dated: \_\_\_\_\_

4/19/02



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**ATTACHMENT A**

**VERSION WITH MARKINGS TO SHOW CHANGES**

**In the Specification:**

Please amend the specification as follows:

[0027] Figure [16] 19: FP-peg-biotin labels serine proteases but not their respective zymogens. Samples of each protein (100 nM) were treated with FP-peg-biotin (4  $\mu$ M) for 1 hour (50 mM Tris, pH 7.2), quenched with 2X SDS-PAGE loading buffer (reducing), and analyzed by SDS-PAGE and blotting with avidin (45 ng protein/lane, bottom two panels). 50X protein stocks were also run on an SDS-PAGE gel and stained with Coomassie blue (2.2  $\mu$ g/lane, top panels). Note that the weak avidin signal seen in the chymotrypsinogen sample upon longer exposures may represent FP-peg-biotin reactivity with trace amounts of contaminating chymotrypsin, which is reported to compose up to 2% of the purchased proenzyme.

[0028] Figure [17] 20: Comparing the proteome reactivities of FP-biotin and FP-peg-biotin. For all of the experiments depicted in this figure, protein samples represent soluble fractions of rat testis (1  $\mu$ g protein/ $\mu$ L). (A) Protein samples were treated with 4  $\mu$ M of either FP-biotin or FP-peg-biotin for 1 hour (50 mM Tris, pH 7.2) and analyzed by SDS-PAGE and blotting with avidin. A pair of 48 kDa serine hydrolases that were robustly labeled by FP-peg-biotin, but not FP-biotin are highlighted (arrowhead). (B-D) Concentration dependence of FP--proteome reactions. Protein samples were treated with 0.5, 1, 2, 4, or 8  $\mu$ M of either FP-biotin (B) or FP-peg-biotin (C) for 1 hour (50 mM Tris, pH 7.2) and analyzed by SDS-PAGE and blotting with avidin. The upper and lower panels represent 1 and 10 minute film exposures respectively. Several serine hydrolases appearing to show no FP-peg-biotin concentration dependence in a one hour reaction (C, arrowheads), exhibited strong probe concentration dependence in a one minute reaction (D, arrowheads).

[0029] Figure [18] 16: pH-dependence of FP-proteome reactions. Protein samples were treated with either FP-biotin (left panel) or FP-peg-biotin (right panel) for 1 hour (50 mM

Tris 50 mM CAPS 50 mM sodium citrate) at pH 6.0, 7.0, 8.0, or 9.0 and analyzed by SDS-PAGE and blotting with avidin. Serine hydrolases displaying pH optima of 8 and 9 are highlighted by triple and double arrowheads, respectively. Proteins exhibiting heat-insensitive FP-reactivity at pH 9 are highlighted by single arrowheads.

[0030] Figure [19] 17: The serine hydrolase activity profile of Triton-solubilized brain membranes is similar to that of unsolubilized brain membranes (reaction conditions: 1  $\mu$ g/ $\mu$ L protein, 2  $\mu$ M FP-biotin, 30 min reaction, 50 mM Tris, pH 8.0, with or without 0.2% Triton X-100).

[0031] Figure [20] 21: (A) Kinetic analysis of FP-proteome reactions. Triton-solubilized brain membranes (1  $\mu$ g protein/ $\mu$ L) was treated with 4  $\mu$ M of either FP-biotin (left panel) or FP-peg-biotin (right panel) for the indicated reaction times, after which the assays were quenched with 2X SDS-PAGE loading buffer and analyzed by SDS-PAGE-avidin blotting. Serine hydrolase activities that reacted at a faster rate with FP-biotin or FP-peg-biotin are highlighted (single and double arrowheads, respectively). The top and bottom panels represent 1 and 5 minute film exposures, respectively. (B) Multiplexing of biotinylated FPs enhances coverage of active serine hydrolases in complex proteomes. Solubilized brain membrane proteins were treated with the indicated concentrations of biotinylated FPs (30 min reaction). Highlighted are three serine hydrolases that are collectively visualized more clearly in an FP-proteome reaction containing a mixture of FP-biotin and FP-peg-biotin than in reactions conducted with each probe alone (bracket).

[0032] Figure [21] 22. A comparison of membrane-associated serine hydrolase activities expressed in a panel of rat tissues. Protein samples (1  $\mu$ g/ $\mu$ L) were treated with FP-biotin (4  $\mu$ M) for 1 hour (50 mM Tris, pH 8.0, 0.2% Triton), quenched with 2X SDS-PAGE loading buffer, and analyzed by SDS-PAGE/avidin blotting on either a 10% (left panels) or 8% (right

panels) polyacrylamide gel. Highlighted are brain-enriched (single arrowheads), heart-enriched (double arrowhead), and testis-enriched (triple arrowhead) serine hydrolase activities.

[0033] Figure [22] 18. Evaluating the target selectivity of a noncovalent serine hydrolase inhibitor in complex proteomes. Competition reactions between varying concentrations of oleoyl trifluoromethyl ketone (OTFMK) and biotinylated FPs identifies several brain membrane serine hydrolases targeted by OTFMK. The arrowhead highlights the brain enzyme fatty acid amide hydrolase (FAAH), a known OTFMK target, and the bracket highlights two additional brain membrane serine hydrolases sensitive to OTFMK.

[0176] A similar scheme was utilized to synthesize biotinylated sulfonate esters for use as ABPs as shown in Figure [4] 9. Similarly, Figure 9 shows a strategy for stereocontrolled synthesis of conformationally well-defined spiroepoxides of type VI (see also, Sornensen, et al., *Angew. Chem. Int. Ed.*, 1999, 38:971-974, herein incorporated by reference in its entirety). Compounds of type VI are analogs of the metalloprotease (MetAp-2) inhibitor fumagillin and are used as ABPs herein.

[0177] To initially test FP-biotin's utility as an activity-based probe for serine hydrolases, we reacted this agent with the mammalian serine amidase, fatty acid amide hydrolase (FAAH) (Cravatt, et al., (1996) *Nature (London)* 384k, 83-87). FP-biotin behaved as a potent irreversible inhibitor of FAAH (data not shown), displaying properties similar to those of other FP inhibitors of the enzyme (Patricelli, et al., *supra*; Deutsch, et al. (1997) *Biochem. Pharmacol.* 53, 255-260). We have shown that serine residue 241 serves as FAAH's catalytic nucleophile and mutation of this residue to alanine (S241A) generates an inactive enzyme (Patricelli, et al., *supra*). Therefore, FP-biotin (2  $\mu$ M) was reacted with both FAAH and the S241A mutant (80 nM) for 10 minutes, after which the proteins were subjected to standard SDS-PAGE-Western blotting procedures using either anti-FAAH antibodies or avidin as detection reagents [(Fig. 1A)]. While anti-FAAH antibodies identified both FAAH and the S241A mutant, avidin detected only FAAH

in the FP-biotin reactions, demonstrating that this inhibitor exclusively reacted with the active form of the enzyme.

[0188] **FP-Peg-Biotin Reacts with Serine Proteases in an Activity-Dependent Manner.** To demonstrate that biotinylated FPs could distinguish active proteases from their inactive zymogens, FP-peg-biotin was incubated with equal amounts of trypsin, trypsinogen, chymotrypsin, and chymotrypsinogen. The products of each reaction were then compared by SDS-PAGE and blotting with avidin (Figure [16] 19). FP-peg-biotin strongly labeled both trypsin and chymotrypsin, but exhibited little or no reactivity with their respective zymogens. Protein staining revealed that a significant fraction of the chymotrypsin sample had been degraded during the course of setting up the reaction. Nonetheless, FP-peg-biotin still showed a much stronger labeling intensity with the chymotrypsin sample than with its chymotrypsinogen counterpart, despite the substantially lower quantity of protein present in the former reaction. Indeed, the low level of FP-peg-biotin labeling observed in the chymotrypsinogen sample (seen only in the 5X exposure panel) may have actually represented reactivity with trace amounts of chymotrypsin rather than with the zymogen itself, as the purchased proenzyme is reported to possess up to 2% active protease.

[0189] **Comparing the Serine Hydrolase Activity Profiles Generated with FP-Biotin and FP-peg-Biotin.** Soluble fractions of rat testis (1  $\mu\text{g}/\mu\text{L}$ ) were treated with either FP-biotin or FP-peg-biotin (4  $\mu\text{M}$ ) for 1 h at room temperature (50 mM Tris, pH 7.2, 150 mM NaCl) and the labeled serine hydrolase activities detected by SDS-PAGE and blotting with avidin. Control reactions in which the proteome was heat-denatured prior to treatment with biotinylated FPs were also analyzed to distinguish specific (heat-sensitive) from nonspecific (heat-insensitive) protein reactivity. The serine hydrolase activity profiles produced by each biotinylated FP were strikingly similar to one another with a single notable exception (Figure [17] 20A). A pair of 50 kDa serine hydrolase activities were strongly labeled by FP-peg-biotin, but displayed very low reactivity with FP-biotin (arrowhead). FP-proteome reactions conducted for longer times (2



hours to overnight) increased the labeling intensity of some serine hydrolases, but did not result in the detection of any new heat-sensitive protein reactivities. Therefore, one hour FP-proteome reactions provided profiles that were considered to represent "maximal coverage" of serine hydrolase activities, and unless otherwise noted, subsequently described reactions were conducted for this length of time.

[0190] The Probe Concentration Dependence of FP-Proteome Reactions. Samples of the rat testis proteome were treated for one hour with either FP-biotin or FP-peg-biotin over a probe concentration range of 0.5 - 8.0  $\mu$ M (Figure [17] 20B and C). Most FP-biotin-labeled proteins displayed enhanced signal intensities with increasing amounts of probe, indicating that their reactivities were not saturated at low micromolar FP-biotin concentrations. In contrast, several of the FP-peg-biotin-labeled proteins showed no detectable change in their signal intensities with increasing concentrations of probe (Figure [17] 20C, arrowheads). These enzymes had either reacted to completion, or were saturated in their rates of labeling at all of the FP-peg-biotin concentrations tested. Kinetic experiments supported the former explanation, as clear concentration-dependent labeling for all of these proteins could be observed in reactions conducted for a shorter time (1 min; Figure [17] 20D, arrowheads).

[0191] Notably, in reactions conducted for one hour with concentrations of 4 - 8  $\mu$ M FP-peg-biotin, at least 18 distinct serine hydrolase activities could be resolved on a single lane of a one-dimensional SDS-PAGE gel (Figure [17] 20C).

[0193] The pH-Dependence of FP-Proteome Reactions. A single mixed buffer assay was used to evaluate the pH-dependence of FP-proteome reactions over a pH range of 6.0-9.0 (conditions: 1  $\mu$ g/ $\mu$ L soluble testis protein, 4  $\mu$ M biotinylated FP, 50 mM Bis-Tris Propane, 50 mM CAPS, 50 mM citrate, 150 mM NaCl; one hour reaction). Several types of pH-dependence were observed among the FP-labeled proteins, with some serine hydrolases exhibiting an optimal FP reactivity at pH 8.0 (Figure [18] 16, triple arrowheads) and others showing an FP reactivity that

continued to increase in intensity as the pH was raised to 9.0 (double arrowheads). Although multiple serine hydrolases appeared to show "pH-independent" FP reactivities, kinetic analyses indicated that most of these enzymes had labeled to completion during the time course of the reactions (see above). Thus, these enzymes likely react with FPs in a pH-dependent manner that might be visualized by modifying the parameters of the reaction to slow their rates of labeling (e.g., lowering the probe concentration and/or reducing the incubation time). Finally, background FP reactivity increased significantly at pH 9.0, with several labeled proteins appearing in the preheated control lane (single arrowheads). Coomassie blue staining revealed that these labeled proteins were all high abundance constituents of the testis proteome, indicating that heat-insensitive labeling represents a nonspecific form of FP reactivity. Considering further that the majority of serine hydrolases displayed similar (or greater) FP reactivities at pH 8.0 relative to pH 9.0, the former pH appears better suited for the functional analysis of this enzyme family in complex proteomes.

[0195] Initially, brain membrane proteins were treated with FP-biotin both prior to and after solubilization with Triton X-100 to examine the effects of detergent on the FP- membrane proteome reaction. Similar serine hydrolase activity profiles were observed with membrane-associated and Triton-solubilized brain protein samples (Figure [19] 17), indicating that detergent solubilization maintained most of the membrane-bound serine hydrolases in a catalytically active state. Therefore, subsequent labeling experiments were conducted with Triton-solubilized brain membrane proteins.

[0196] Most brain membrane serine hydrolases exhibited rates of FP reactivity that could be monitored over a time course of 1-60 minutes under the following set of reaction conditions: 4  $\mu$ M biotinylated FP, 1  $\mu$ g/ $\mu$ L protein, 50 mM Tris buffer, pH 8.0, with 0.2% Triton X-100. Interestingly, examples were observed for each of the three potential types of serine hydrolase reactivity profiles: 1) enzymes that labeled with FP-biotin at faster rates than FP-peg-biotin (Figure [20] 21A, single arrowhead), 2) enzymes that labeled with FP-peg-biotin at faster rates



than FP-biotin (double arrowheads), and 3) enzymes that labeled with FP-biotin and FP-peg-biotin at similar rates (triple arrowheads). In particular, a 65 kDa SH activity was apparently labeled to completion with FP-biotin within one minute (Figure [20] 21A, left panel), but reacted at a much slower rate with FP-peg-biotin (its signal intensity still increasing from 30 to 60 minutes; Figure [20] 21A, right panel). We suspected that to demonstrate that this serine hydrolase represents fatty acid amide hydrolase (FAAH), a brain integral membrane enzyme that displays a strong preference for long aliphatic amide substrates, we treated brain membrane proteins with each biotinylated FP and measured FAAH catalytic activity at two time points during the reaction. No FAAH activity could be detected in the FP-biotin treated samples after incubations for either 10 or 60 minutes. In contrast, significant FAAH activity was observed in the FP-peg-biotin treated samples at both time points, with the 10 and 60 minute incubations displaying 60% and 30% FAAH activity, respectively (relative to an untreated control sample). Thus, FAAH was inactivated at a much faster rate by FP-biotin than FP-peg-biotin, consistent with this enzyme's substrate selectivity.

[0197] Considering that several brain membrane serine hydrolases reacted preferentially with one biotinylated FP over the other, we showed that by treating a single proteome with a mixture of both probes, a more complete serine hydrolase activity profile is obtained. Treating brain membrane proteins with 2  $\mu$ M each of FP-biotin and FP-peg-biotin provided a serine hydrolase activity profile that resembled closely the predicted merger of profiles generated with each FP individually (Figure [20] 21B). These data indicate that adding multiple activity-based probes to a single proteome ("multiplexing") can enhance the coverage of active enzymes present in that sample.

[0198] Comparing the Membrane-Associated Serine Hydrolase Activity Profiles from Different Rat Tissues. Having observed the large number of serine hydrolases associated with brain membranes, we compared the serine hydrolase activity profiles of membrane fractions from a panel of rat tissues. Each membrane fraction was first washed with 1 M NaCl prior to

solubilization of its protein content with Triton X-100. This protocol was selected to enrich for integral membrane proteins, a class of proteins notoriously resistant to analysis by standard proteomics methods. Interestingly, each of the tissues examined possessed a unique and complex profile of membrane-associated serine hydrolase activities (Figure [21] 22). Notably, a set of three 48-52 kDa serine hydrolase activities were enriched in brain membranes (single arrowhead). Likewise, heart-enriched and testis-enriched serine hydrolase activities were also observed (double and triple arrowheads, respectively).

[0199] Evaluating the Target Selectivity of Noncovalent Serine Hydrolase Inhibitors. Biotinylated FPs not only distinguish active serine hydrolases from zymogens (Figure [16] 19), but also from inhibitor-bound enzymes. To demonstrate the target selectivity of serine hydrolase inhibitors directly in complex proteomes, each biotinylated FP was added to a brain membrane proteome treated with varying concentrations of oleoyl trifluoromethyl ketone (OTFMK), a previously described noncovalent FAAH inhibitor. After a 30 minute incubation, the reactions were quenched and analyzed by SDS-PAGE and blotting with avidin. Several serine hydrolases displayed reduced FP-reactivity in the presence of increasing concentrations of OTFMK (Figure 22) indicating that this electrophilic ketone was not only an effective inhibitor of FAAH, but of other brain membrane serine hydrolases as well. Film densitometry was used to estimate OTFMK's potency as an inhibitor of each of its serine hydrolase targets. A pair of 50 kDa serine hydrolases displayed approximately 40 and 80% reductions in both their FP-biotin and FP-peg-biotin reactivities in the presence of 50 and 200  $\mu$ M OTFMK, respectively (bracket). In contrast, FAAH's FP-peg-biotin reactivity was reduced by approximately 30, 90, and 95% in the presence of 5, 50, and 200  $\mu$ M OTFMK, respectively (arrowhead). These data reveal that OTFMK displays a relatively modest (approximately one order of magnitude) selectivity for FAAH among other brain membrane serine hydrolases. Although both 50 and 200  $\mu$ M concentrations of OTFMK blocked greater than 90% of FAAH's FP-peg-biotin reactivity, this enzyme's FP-biotin reactivity was only weakly affected (~20% reduction) and partially affected (~70% reduction) by 50  $\mu$ M and 200  $\mu$ M OTFMK, respectively. These apparently conflicting data can be rationalized as follows. Of the three observed OTFMK-sensitive serine hydrolases, exclusively FAAH

reacted with FP-biotin at a rate that was too fast to monitor under the reaction conditions employed [(Figure 19A, left panel)]. In such a case, the binding of a reversible inhibitor will only be detected if the inhibitor reduces its protein target's rate of FP reactivity to the extent that the protein no longer labels to completion during the time course of the reaction. Thus, FAAH's sensitivity to OTFMK was likely underestimated in the 30 minute FP-biotin competition assay. On the other hand, FP-peg-biotin labeled FAAH at a rate slow enough to be followed over a 60 minute time course [(Figure 19A, right panel)], and therefore reactions conducted with this probe provided a more accurate assessment of the sensitivity of FAAH to competitive active site-directed agents. Collectively, these results demonstrate that biotinylated FPs can, in cases where they display discernible labeling kinetics, identify the serine hydrolase targets of noncovalent inhibitors directly in complex proteomes.

**[0200] Affinity Isolation and Molecular Characterization of FP-Labeled Serine Hydrolases.**

For activity-based probes like the biotinylated FPs to be of lasting use to proteomics research, these reagents must not only serve as tools for protein detection, but also for protein isolation and identification. In the course of attempting to affinity purify FP-biotinylated proteins by avidin agarose chromatography, we noted that several labeled proteins failed to bind the avidin matrix in their native state (Figure [8] 23A, arrowheads). However, if protein samples were denatured prior to treatment with avidin beads, efficient depletion of all of the biotinylated proteins was achieved. Subsequent washing of the beads and elution with SDS-PAGE loading buffer provided a sample greatly enriched for biotinylated proteins (Figure [8] 23A, right panel). The elution sample was run on an SDS-PAGE gel and the biotinylated proteins excised from the gel, digested with trypsin, and the resulting peptide mixtures analyzed by MALDI mass spectrometry. This one-step isolation method identified seven of the labeled serine hydrolases present in a soluble rat testis proteome as: acylpeptide hydrolase (82 kDa, accession #CAA33040), prolyl oligopeptidase (80 kDa, BAA25544), carboxylesterase 1 (80 kDa, accession #JX0054), carboxylesterase 10 (60 kDa, accession #P16303), long chain acyl CoA hydrolase (48 kDa, accession #088267), platelet-activating factor (PAF) acetylhydrolase a1 subunit (32 kDa, accession #NP\_032802), and PAF acetylhydrolase a2 subunit (30 kDa,

accession #035264) (Figure 23B). An additional 45 kDa serine hydrolase activity provided a MALDI tryptic peptide map that did not match those of any proteins in the public databases. Analysis of the electrospray MS fragmentation pattern of one of the tryptic peptides from this protein ( $M + H^+ = 1099$  Da) provided the following sequence information: GFVVAAIEHR. BLAST database searches with this peptide sequence identified 80 and 90% identical sequences in the human and dog versions, respectively, of a 45 kDa serine hydrolase referred to as plasma PAF acetylhydrolase or LDL-associated phospholipase A2 (human protein accession #AAB04170.1). Thus, the isolated 45 kDa FP-biotin-reactive testis protein was likely a novel rodent member of this family of secreted serine hydrolases.

[0228] The following features of the sulfonate-proteome reaction were varied in order to test their influence on the observed specific and nonspecific protein labeling patterns: time, sulfonate concentration, pH, and the presence/absence of scavenging nucleophiles. For these studies, the reactivity of pyridylsulfonate 1 with the testis proteome was examined. The two testis proteins specifically targeted by 1 were labeled at similar rates, with their signal intensities increasing from 5-40 minutes and then plateauing from 40-120 minutes (Figure [13] 7A). The absence of additional reactivity from 40-120 minutes could signify that the proteins had labeled to completion by 40 minutes, or less likely, that the concentration of 1 in the reaction was significantly depleted by these later time.

[0229] Sulfonate 1's specific and nonspecific proteome reactivities were evaluated over a range of probe concentrations (1-50  $\mu$ M). From 1-10  $\mu$ M, sulfonate 1 showed specific reactivity with the 31 and 55 kDa proteins that increased in intensity with increasing concentrations of reagent (Figure [13] 7B). Over this concentration range, sulfonate 1 displayed very low levels of heat-insensitive reactivity with the proteome. From 10 to 50  $\mu$ M of 1, the signal intensity of the 31 kDa protein continued to increase, while the intensity of the 55 kDa protein remained relatively constant. Over this concentration range, sulfonate 1's nonspecific labeling increased dramatically, especially in the higher molecular mass range where most of the abundant testis proteins reside. Importantly, however, no new specifically labeled protein targets were identified

over this concentration range. Thus, a concentration range of 5-10  $\mu$ M appeared optimal for maximizing sulfonate 1's specific versus nonspecific proteome reactivity.

[0230] The nonspecific and specific proteome reactivities of sulfonate 1 showed different pH-dependencies, with the former appearing as an inverted bell-shape curve (higher background labeling at pH 6 and 9 than at pH 7 and 8) and the latter increasing in intensity from pH 6 to 8 and plateauing from pH 8 to 10 (Figure [13] 7C, D). Thus, reactions conducted at pH 7 and 8 produced the highest level of specific reactivity, while at the same time resulting in the lowest degree of nonspecific reactivity.

[0231] Sulfonate 1's intrinsic reactivity with nucleophiles was examined by conducting proteome reactions in the presence of millimolar concentrations of free thiols (glutathione,  $\alpha$ -mercaptoethanol, or dithiothreitol). If this sulfonate displayed a high reactivity with generic nucleophiles, then the probe's effective concentration in thiol-treated proteome reactions should be greatly reduced, resulting in a significant decrease in the signal intensity of specifically labeled proteins. However, none of the tested thiols affected the labeling intensity of the 55 kDa protein, indicating that sulfonate 1's intrinsic reactivity with nucleophiles is low (Figure [13] 7D). In contrast, a moderate decrease in the labeling intensity of the 31 kDa protein was detected in the presence of free thiols.

[0233] A tissue blot with sulfonate 1 revealed that the labeled 55 kDa protein was most abundant in soluble fractions of rat liver, and therefore the protein was purified from this source. The 55 kDa protein was partially purified by Q-Sepharose anion exchange chromatography. Aliquots of the flow-through and elution fractions of this column were labeled with 1, and the 55 kDa protein was identified in the flow-through fractions. These fractions were combined, labeled with 5  $\mu$ M 1 for 30 min, and the protein separated from excess sulfonate by size exclusion chromatography. The protein sample was then treated with avidin agarose beads to isolate the 1-labeled 55 kDa protein. Elution of the avidin-bound proteins was achieved by



adding one volume of standard SDS-PAGE loading buffer and heating (90°C, 5 min). This avidin-based affinity purification procedure provided a highly concentrated sample of the 55 kDa protein that was separated by SDS-PAGE and either blotted with avidin (Figure [14] 13A) or stained with Coomassie blue. The 55 kDa protein was excised from the stained gel, treated with trypsin, and the resulting peptide mixture analyzed by MALDI-TOF mass spectrometry. MS-FIT and Profound searches of protein databases identified the protein as cytosolic 2 class I aldehyde dehydrogenase (cALDH-I; nine tryptic peptides ranging from 1189 to 2055 Da matched this enzyme, 50% total sequence coverage; Figure [14] 13A), a member of a superfamily of NAD<sup>+</sup>-dependent enzymes responsible for the oxidation of endogenous and exogenous aldehydes to carboxylic acids [Wang, et al. (1996) J. Biol. Chem. 271, 16288-93; Yoshida, et al. (1998) Eur. J. Biochem. 251, 549-57].

[0234] In order to confirm the specific reactivity of cALDH-I with sulfonate 1, this protein was recombinantly expressed in both eukaryotic and prokaryotic systems. The cALDH-I cDNA was subcloned into the pcDNA3 mammalian expression vector and then transfected into COS-7 and MCF-7 cells. cALDH-I-transfected COS-7 and MCF-7 cells both expressed a 55-kDa protein that labeled strongly with sulfonate 1 (Figure [14] 13B). In contrast, this sulfonate-reactive protein was not detected in mock-transfected versions of each cell type. cALDH-I was also recombinantly expressed in *E. coli* using the pTrcHis system. Lysates from cALDH-I-transformed *E. coli* were treated with sulfonate 1 and found to express a single reactive protein of the predicted size for the cALDH-I enzyme bearing an N-terminal histidine tag (60 kDa; Figure [14] 13C). The His-tagged cALDH-I was purified from *E. coli* lysates by sequential metal affinity and gel filtration chromatography. This prokaryotic expression system routinely provided 15 mg/L culture volume of purified cALDH-I enzyme.

[0238] Analogs of 15 were synthesized in which the agent's octyl and pyridyl substituents were replaced with ethyl (16) and methyl (17) groups, respectively. cALDH-I was incubated for 60 minutes with 50 µM of either 15, 16, or 17, and the percentage of cALDH-I activity



remaining was determined (Table 2). While 15 completely inactivated cALDH-I under these conditions, 16 and 17 produced weak and no inhibition, respectively. A  $K_{obs}/[I]$  value of 0.25 M<sup>-1</sup> min<sup>-1</sup> was calculated for 16, representing a second order inhibition rate constant 40000 times lower than that determined for 15. 15 – 17 were also tested for their ability to block sulfonate 1's reactivity with cALDH-I in the soluble testis proteome. The testis proteome was preincubated for 30 min with each nonbiotinylated sulfonate at concentrations of 5 or 50  $\mu$ M. The proteome samples were then treated with 5  $\mu$ M 1 and the reaction mixtures incubated for 30 minutes prior to analysis by SDS-PAGE and avidin blotting. Consistent with the inhibition kinetics described above, only 15 blocked the labeling of cALDH-I by 1 in the testis proteome (Figure [15] 14B). Thus, the structure-activity relationship (SAR) determined for sulfonate inactivation of purified cALDH-I correlated well with the SAR observed for sulfonate labeling of this enzyme in complex proteomes. Collectively, these data highlight that the interaction of 15 (and by extrapolation 1) with cALDH-I depends on both the aryl and alkyl chain groups of the inhibitor's structure.

[0240] The rat testis proteome was treated with either 2.5  $\mu$ M sulfonate 1, 5  $\mu$ M FP-biotin, or a mixture of 2.5  $\mu$ M 1 and 5  $\mu$ M FP-biotin [Liu, et al. Proc. Natl. Acad. Sci USA 96, 14694-99], and the resulting heat-sensitive labeling profiles were visualized by SDS-PAGE and avidin blotting. As can be seen in Figure [17] 15, applying a mixture of sulfonate 1 and FP-biotin to the testis proteome effectively detected in a single sample the proteins labeled by both probes individually. Notably, a preheated sample treated with the same probe mixture displayed a very low level of labeling that was comparable to the nonspecific reactivity observed when each probe was tested alone. These data support that by multiplexing ABPs, one can significantly increase the coverage of specific protein reactivities detectable in a single proteome assay.

In addition, there appear to be some clerical or typographical errors in the Examples.

They seem to be of a nature that would be apparent to one of ordinary skill in the art, but, like the figure references, it may be worthwhile to correct them to make it easier for the patent to be read after it is issued.

**[0152]** The following examples provide an illustrative synthetic scheme for a fluorophosphonates-based ABP which includes a biotin label or tag. These "FP-biotin" ABPs are in no way limiting and are merely illustrative. One of skill in the art can use standard methods to design other ABPs as described herein.

On page 62, before paragraph 154, insert a title of the Example: "PREPARATION OF FP-PEG-BIOTIN."

**In the claims:**

Please amend the claims as follows:

11. (Amended) A method for determining in a proteomic mixture the presence of active target members of a group of related proteins, said related proteins related in having a common functionality for conjugation at an active site, said method comprising:

combining a first portion of said proteomic mixture [in wild type form] with at least one activity-based probe comprising a reactive functionality specific for said active site when active, under conditions for conjugation of said probe to said target members;

combining a second portion of said proteomic mixture that has been subjected to [after] non-specific [deactivation] inactivation with said probe(s) under [said] the same conditions used with said first portion of said proteomic mixture;

determining the presence of target members conjugated with said probe(s) in each of said first and second portions of proteomic mixtures [in active and inactive form] ; whereby the presence of a greater amount of target members conjugated to said probe(s) in said first portion of said proteomic mixture than in said second, inactivated portion of said proteomic mixture indicates the presence of an active target member [when said target members are conjugated to target members in said proteomic mixture in active form and in less amount in inactive form, the presence of active members is determined].

12. A method according to Claim 11, comprising the additional step of characterizing said active target members conjugated with said probe(s) [in active form and in less amount in inactive form].

13. A method according to Claim 12, wherein said characterizing comprises degrading said active target member and determining the resulting fractions by mass spectrometry.

14. A method according to Claim 11, employing a plurality of activity-based probes having different reactive functionalities specific for different groups of related proteins.

15. A method according to Claim 11, wherein said activity-based probe(s) comprises a detectable label.

16. A method according to Claim 11, wherein said proteomic mixture is in an intact cell.

17. A method for determining in a plurality of proteomic mixtures the presence of active target members of a group of related proteins in each of said proteomic mixtures, said related proteins related in having a common functionality for conjugation at an active site, said method comprising:

combining each of said proteomic mixtures [in wild type form] with at least one activity-based probe comprising a reactive functionality specific for said active site when active, under conditions for conjugation of said probe(s) to said target members;

determining the presence of target members conjugated with said probe in each of said proteomic mixtures;

[analyzing for the presence of target members conjugated with said probe using simultaneous individual capillary electrokinetic analysis or capillary HPLC;]

whereby the presence of [when] said target members [are] conjugated to said probe(s) [target members] in said proteomic mixtures is indicative of the presence of active target members in said mixtures [is determined].

18. A method for determining in a plurality of proteomic mixtures the presence of active target members of a group of related proteins, said related proteins related in having a common functionality for conjugation at an active site, said method comprising [according to Claim 17 including the additional steps of]:

combining a first portion of each of said proteomic mixtures with at least one activity-based probe comprising a reactive functionality specific for said active site when active, under conditions for conjugation of said probe(s) to said target members;

combining a second portion of each of said proteomic mixture that has been subjected to non-specific deactivation, with said probe(s) under the same conditions used with said first portion of said proteomic mixture; and [inactivating a portion of said proteomic mixture;

combining said inactivated proteomic mixture with said probe under conditions for conjugation;

analyzing] determining [for] the presence of target members conjugated with said probe(s) in each of said first and second portions of said [inactivated] proteomic mixtures;  
[and

rejecting conjugates from said wild type proteomic mixture in less amount than the amount of conjugate from said inactivated mixture]

whereby the presence of a greater amount of target members conjugated to said probe(s) in said first portion of said proteomic mixtures than in said second, inactivated portion of said proteomic mixtures indicates the presence of active target members.

19. A method for determining in a proteomic mixture the presence of active target members of a group of related enzymes, said related enzymes related in having a common functionality for conjugation at an active site, said method comprising:

combining a first portion of said proteomic mixture [in wild type form] with at least one activity-based probe comprising a reactive functionality specific for said active site when active, under the same conditions for conjugation of said probe to said target members;

combining a second portion of said proteomic mixture that has been subjected to [after] non-specific in[de]activation, with said probe(s) under the [said] same conditions used with said first portion of said proteomic mixture;

determining the presence of target members conjugated with said probe(s) in each of said first and second portions of said proteomic mixture[s in active and inactive form];

whereby the presence of a greater amount of target members conjugated to said probe(s) in said first portion of said proteomic mixture than in said second, inactivated portion of said proteomic mixture indicates the presence of an active target member [when said probe is conjugated to at least one target member in said proteomic mixture in active form and in lesser amount in inactive form, the presence of active members is determined].

20. A method according to Claim 19, wherein said probe comprises a ligand and said determining is by binding said ligand in a conjugate to a support and isolating said conjugate.

21. A method according to Claim 19, wherein said reactive functionality is a fluorophosphonate or fluorophosphoroate [and said enzymes are serine hydrolases].

22. A method according to Claim 19, wherein [related enzymes are cysteine hydrolases and] said active functionality is an  $\alpha$ -haloketone.

23. A method according to Claim 19, wherein [related enzymes are enzymes comprising at least one of cysteine, histidine, aspartate and glutamate at said active site and] said active functionality is sulfonate ester or epoxide.

24. A method according to Claim 19, wherein [related enzymes are aldehyde dehydrogenases and] said active functionality is a sulfonate ester.

25. A method according to Claim 19, wherein [related enzymes are metalloenzymes and] said active functionality is  $\alpha$ -halohydroxamic acid.

26. A method according to Claim 19, wherein [related enzymes are redox enzymes and] said active functionality is an alkyne.



27. (New) A method according to Claim 17 or 18, comprising the additional step of characterizing said active target members conjugated with said probe(s) .
28. (New) A method according to Claim 27, wherein said characterizing comprises degrading said active target member and determining the resulting fractions by mass spectrometry.
29. (New) A method according to Claim 17 or 18, employing a plurality of activity-based probes having different reactive functionalities specific for different groups of related proteins.
30. (New) A method according to Claim 17 or 18, wherein said activity-based probe(s) comprises a detectable label.
31. (New) A method according to Claim 17 or 18, wherein said proteomic mixture is in an intact cell.
32. (New) A method according to Claim 17 or 18 further comprising the step of analyzing for the presence of proteins conjugated with said probe(s) using simultaneous individual capillary electrokinetic analysis or capillary HPLC.
33. (New) A method according to Claim 11, 17, 18 or 19 wherein said activity-based probe(s) are of the formula:
- $$R^* (F - L) - X$$
- wherein:
- X is a ligand for binding to a reciprocal receptor or a chemically reactive functionality for reacting with a reciprocal functionality to add a ligand;
- L is a linking group;
- F is a functional group reactive at an active site of a target enzyme; and
- R is H or a moiety of less than 1kDal providing specific affinity for said target enzymes;
- the \* intends that R is a part of F or L.

34. (New) A method according to Claim 33, wherein F is a sulphonyl group and R is other than H and bonded to F.

35. (New) A method according to Claim 33, wherein F is a fluorophosphonyl or fluorophosphoryl group.

36. (New) A method according to Claim 11, wherein at least one of L and X comprise at least one isotope in unnatural amount, and including the additional step of:  
releasing at least a portion of said probe from said conjugate and identifying said portion by means of isotopic difference.

37. (New) A method according to any of Claims 11-13, 15-21, 27, 28, 30-33, 35 or 36 wherein said activity-based probe(s) are FP-biotin.

38. (New) A method according to any of Claims 11-13, 15-21, 27, 28, 30-33, 35 or 36 wherein said activity-based probe(s) are FP-peg-biotin.

39. (New) A method according to any of Claims 11-13, 15-20, 23, 24, 27, 28, 30-34 or 36 wherein said activity-based probe(s) are selected from the group consisting of Sulfonate 1 – Sulfonate 17.

40. (New) A method according to Claim 39 wherein said activity-based probe(s) are Sulfonate 15.

41. (New) A method according to Claim 14 or 29 wherein said activity-based probe(s) are selected from the group consisting of FP-biotin, FP-peg-biotin and Sulfonate 1 Sulfonate 17.

42. (New) A method according to Claim 21 wherein said group of related enzymes is serine hydrolases.
43. (New) A method according to Claim 22 wherein said group of related enzymes is cysteine hydrolases.
44. (New) A method according to Claim 23 wherein said group of related enzymes is related in having a common functionality comprising at least one of the following: cysteine, histidine, aspartate, and glutamate.
45. (New) A method according to Claim 24 wherein said group of related enzymes is alcohol dehydrogenases.
46. (New) A method according to Claim 25 wherein said group of related enzymes is metalloenzymes.
47. (New) A method according to Claim 26 wherein said group of related enzymes is redox enzymes.

REMARKS

Group IV (Claims 17 and 18) relates to methods for determining in a plurality of proteomic mixtures, the presence of active target members of a group of related proteins. Group III (Claims 11-16) relates to a method for determining in a proteomic mixture, the presence of active target members of a group of related proteins. Group V (Claims 19-26) relates to methods for determining in a proteomic mixture, the presence of active target members of a group of related enzymes.

Thus, Groups IV and III are related in that they both relate to methods of determining the presence of active target members of a group of related proteins. Groups IV relates to methods involving a plurality of proteomic mixtures, while Group III relates to methods involving a proteomic mixture. It would appear to pose no greater burden on the Examiner to search the claims of Group III (plurality of proteomic mixtures) along with those of Group IV (a proteomic mixture). Similarly, Groups IV and V are related in that they both relate to methods of determining in a proteomic mixture the presence of active target members of a group of related proteins. As is well known in the art, enzymes are proteins (see, e.g., the specification at page 16, lines 9-17 and page 31, lines 2-6), and for this reason, it would not involve any additional searching efforts on the part of the Examiner to perform a search for Group V in addition to Group IV.

Although the Examiner alleges that the claims of each Group are independent and patentably distinct, it is submitted that it would not be a serious burden on the Examiner to examine the claims together (see MPEP 803). Although the Groups of claims are not classified in the same Class, it is submitted that it would not be a serious burden on the Examiner to examine the claims of Groups III, IV and V together because the same method would be utilized to determine the presence of an active target member. In fact, since an enzyme is a species of protein, art pertaining to Group V would necessarily need to be considered in searching Groups III and IV. As such, it is submitted that a thorough search of the claims of Groups III, IV and Group V would reveal art relevant to all three Groups. Under MPEP 808.2, Applicants submit that the restriction is not proper. Accordingly, it is respectfully requested that the Examiner reconsider the restriction requirement with respect to the claims of Groups III, IV and V and examine the claims together.

Finally, although Applicants traverse the restriction requirement for the reasons set forth above, Applicants nevertheless elect the claims of Group IV, claims 17 and 18, for examination in order to be fully responsive to the Communication.

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No fee is deemed necessary in connection with the filing of this Response to Restriction Requirement. However, if any fee is required, authorization is hereby given to charge the amount of any such fee, or credit any overpayment, to Deposit Account No. 50-1355.

Respectfully submitted,

Date: 4/19/02



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